

AD _____

Award Number: DAMD17-01-1-0777

TITLE: Fundamental Patterns Underlying Neurotoxicity Revealed by
DNA Microarray Expression Profiling

PRINCIPAL INVESTIGATOR: Karen L. O'Malley, Ph.D.

CONTRACTING ORGANIZATION: Washington University School of Medicine
St. Louis, Missouri 63110-1093

REPORT DATE: September 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 01 - 31 Aug 02)	
4. TITLE AND SUBTITLE Fundamental Patterns Underlying Neurotoxicity Revealed by DNA Microarray Expression Profiling			5. FUNDING NUMBERS DAMD17-01-1-0777	
6. AUTHOR(S) Karen L. O'Malley, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Washington University School of Medicine St. Louis, Missouri 63110-1093 E-Mail: omalleyk@pcg.wustl.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains color.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The selective neurotoxins 1-methyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) have been widely used to generate animal models of Parkinson's Disease (PD). To understand and order the genetic events associated with these neurotoxins, DNA microarray technology has been used to monitor differences in gene expression patterns in normal versus pathological conditions. Eleven thousand murine genes and expressed sequence tags were screened to determine changes in gene expression caused by MPP+, the active metabolite of MPTP, and 6-OHDA in a mouse CNS dopaminergic cell line. These studies were facilitated by an in-house GeneChip Facility providing microarrays, services and software. Data derived from either toxin paradigm was compared to identify transcriptional changes associated with parkinsonism-inducing neurotoxins. Present findings indicate that both of these neurotoxins induce ER stress although not to the same degree. Identification of key genetic components of this response may suggest new points of intervention. Taken together, these experiments will help clarify the molecular mechanisms associated with 6-OHDA and MPP+ toxicity and might aid in developing novel therapeutic avenues to pursue relevant to PD.				
14. SUBJECT TERMS MPP+, 6-OHDA, neurotoxicity, gene expressing profiling, DNA microarray expression profiling			15. NUMBER OF PAGES 13	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

20030122 049

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	7
References.....	8
Appendices.....	

Introduction

Parkinson's Disease is a neurodegenerative disorder of unknown etiology associated with the loss of the dopaminergic cells in the substantia nigra. The selective neurotoxins MPTP and 6-OHDA have been widely used to generate animal models of PD although it is still not known how dopaminergic neurons die in response to these toxins. To test the hypothesis that MPTP and 6-OHDA induce unique patterns of gene expression in the CNS-derived dopaminergic cell line MN9D, DNA micro array analyses is being performed from various toxin-treated time points. Both reverse transcription/PCR amplification and western blots are being used to verify changes in selected subsets of differentially regulated transcripts. Selected transcripts are also being tested for toxin-induced changes in primary cultured dopaminergic neurons. Data from both toxin paradigms are being compared with each other to identify shared changes in gene expression. These data will also be compared with data available from other apoptotic or necrotic models. Just as studies in other model systems have uncovered novel signaling pathways, these experiments are also revealing unanticipated pathways that contribute to MPP+ and 6-OHDA neurotoxicity. Taken together, these large-scale gene expression analyses will provide a wealth of information about the role and mode of regulation of genes involved in pathological models of PD.

Body

A. Does the neurotoxin MPP+ differentially regulate sets of genes?

To test the hypothesis that MPP+ alters gene transcription as part of its neurotoxic program, a time course study using cycloheximide to block MPP+ toxicity, was performed. Cells were treated with 50 μ M MPP+ with 10 μ M cycloheximide being added for varying periods of time. The point at which about 50 % of the cells were rescued by blocking protein synthesis was chosen as the best time point at which to harvest RNA. According to Figure1, that time point was 9 hours following MPP+ treatment.

In consultation with experts from our onsite Affymetrix gene chip core facility, we subsequently designed our experiments such that a minimum of 3 separate experiments were performed in which cells were treated with MPP+ for 9 hours and then harvested for RNA preparation at that time point. Cell death was verified in each case by independent experiments done on sibling cultures. RNAs from all three experiments were pooled to form an RNA resource that would minimize experimental variation.

RNA sample preparation was done according to protocols devised by Affymetrix to achieve the best results, particularly for mammalian cells. Briefly, total RNA was prepared from cells using a Qiagen RN-easy kit. Poly (A)+ RNA was prepared from the latter using oligo (dT)-based strategies (Qiagen kit). Two micrograms mRNA was then used to prepare double-stranded cDNA using a T7- (dT) primer containing a T7 RNA polymerase site. Biotin-labeled complementary RNA was produced using the ENZO Bioarray High Yield transcript labeling kit incorporating biotinylated CTP and UTP. Labeled cRNA was then fragmented into sizes from approximately 50-100 bp. Ten micrograms of fragmented sample RNA was then hybridized to an Affymetrix micro array for 16 hours at 45°C.

The Division of Laboratory Medicine at Washington University School of Medicine has established a DNA array facility based on the Affymetrix GeneChip Platform. These chips are available at a discounted fee and the Center provides hybridization, scanning, and discounted software services. Investigators provide the labeled and fragmented cRNA for hybridization. Because MN9D cells are derived from murine mesencephalon, we utilized the mouse gene chips that consist of a 2-chip set containing 11,000 known genes and 800 EST clusters.

Following hybridization, arrays are washed on an Affymetrix fluidics station and stained with streptavidin-phycoerythrin and streptavidin R-phycoerythrin (Molecular Probes). Arrays are again extensively washed and then scanned on a Hewlett-Packard laser confocal GeneArray Scanner. Data obtained was subsequently analyzed using the accompanying Affymetrix GeneChip 3.2 software.

The data obtained for MPP+ is a compiled value from three separate experiments done in triplicate as described above. The expression level of each probe set was plotted to determine the reproducibility of the array-based hybridization signals and to compare gene expression levels by MN9D cells treated with and without MPP+. The ratio of gene intensity in toxin-treated cells to that in control samples was used to represent the toxin-mediated induction. The reciprocal ratio represented repression. Genes were considered up or down-regulated if the fold change was at least 2.0 in individual experiments as well as in averaged, triplicated experiments. These limits are in general agreement with most gene chip experiments.

Figure 2 presents an overview of the data obtained for MPP+-treated cells compared to 6-OHDA-treated cells at the nine hour time point. Overall, in the case of MPP+, very few genes changed at this time point. Mostly there was a general slide in transcriptional response, which is in keeping with numerous studies indicating that MPP+ can lead to decreases in protein and RNA synthesis.

Of interest however was the up regulation of the RNA for Chop10. Chop 10 has been shown previously to be regulated by a variety of stresses including unfolded protein response, growth arrest, DNA damage, amino acid and glucose deprivation, hypoxia, etc. The connection with ER stress is of particular interest since Parkin, the gene associated with AR-JP, has now been shown to function as an E3 ubiquitin-protein ligase (Shimura et al., 2000). Thus loss of Parkin function may lead to the accumulation of substrates that are toxic to dopaminergic neurons. Several Parkin interacting proteins have been identified based on yeast two-hybrid approaches including Pael-R, a G-protein coupled receptor with homology to endothelin receptor type B (Imai et al., 2001). Parkin mutants fail to ubiquitinate Pael-R leading to its accumulation and ensuing unfolded protein response/ER stress-mediated cell death (Imai et al., 2001). Thus unfolded protein response/ER stress may contribute to PD.

B. Does the neurotoxin 6-OHDA differentially regulate sets of genes?

To test the hypothesis that 6-OHDA neurotoxicity alters fundamental patterns of gene expression, experiments were conducted exactly as described above for MPP+ (Figures 1, 2). Data sets obtained from the 6-OHDA experiments are being compared with those derived from MPP+ toxicity as well as other neurodegenerative models.

Because 6-OHDA appeared to induce apoptosis in this model system as well as in primary cultured neurons (Oh et al., 1995; Lotharius et al., 1999; Choi et al., 1999), we anticipated the identification of functional clusters of neurotoxin-responsive genes that would overlap with apoptotic patterns observed in other models. Surprisingly, however, many of the genes that were up regulated were again members of the unfolded protein response. Indeed, Chop 10 induction was even more pronounced in 6-OHDA treated cells than in MPP+ (Figure 2).

C. Verification in MN9D Cells

To verify induction or repression by an independent method, a subset of the most interesting differentially regulated genes were examined by RT/PCR. Because both neurotoxins appeared to induce markers of unfolded protein response, we focused on these genes first. As shown in Figures 3-5, many markers of this pathway were up regulated in response to 6-OHDA treatment including Chop 10. These data support the notion that ER stress may play an important component in PD. Interestingly, MPP+ induced far fewer markers of this pathway suggesting that it may participate with a subset of ER stress response genes.

To determine whether the effects seen at the transcript level (Figure 4) were also reflected at the protein level, identified genes for which antibodies were available were examined for changes in protein expression. Briefly, sibling cultures treated with either 6-OHDA or MPP⁺ were harvested at the same points employed for the micro array measurements. Cells were lysed in standard protein lysate buffer together with proteolytic inhibitors. Lysates were standardized and equal amounts of protein electrophoresed and subsequently immunoblotted. Blots were probed with antibodies directed towards the selected subset of proteins implicated in ER stress. As shown in Figure 5, many of the transcripts implicated in ER stress were also up regulated at the protein level. Moreover, pathways ascribed to this response were also activated as evidenced by predicted increases in phosphorylation. Taken together these data provide strong evidence for both neurotoxins inducing ER stress in this dopaminergic cell line.

D. Are neurotoxin-mediated changes in gene expression recapitulated in cultured dopaminergic neurons?

To confirm and extend results obtained using the dopaminergic cell line model, we are using primary cultures of dopaminergic neurons. The advantages of using this paradigm include the ease of preparation and culture manipulation and the well-documented similarity in responses (Oh et al., 1995; Lotharius et al., 1999). To verify induction or repression of selected subsets of differentially expressed genes in the primary culture model we are using immunocytochemistry to visualize tyrosine hydroxylase, a marker for dopaminergic neurons together with antibodies for the ER stress proteins identified above. These techniques will allow us to confirm relative changes in differentially regulated genes determined by expression profiling in our established dopaminergic culture system. Additionally we are using western blots of lysates harvested from identical cultures at various time points after toxin treatment. Preliminary results indicate that 6-OHDA also induces Chop 10 in primary cultures of dopaminergic neurons. Given these broad similarities in responses, it seems

reasonable to propose that MN9D cells can serve as a homogeneous, renewable resource facilitating studies examining toxin-induced changes in gene expression.

Key Research Accomplishments

- Established time course of toxin-mediated loss of viability together with cycloheximide rescue for MPP⁺ and 6-OHDA.
- Prepared mRNA from normal, 6-OHDA and MPP⁺-treated dopaminergic cells at chosen time point post treatment.
- Determined hybridization patterns of normal and toxin-treated cRNAs using in-house GeneChip Facility and Affymetrix 11,000 gene chip set.
- Analyzed gene expression data.
- Verified differential regulation of particular gene subsets using RNA and western blot analysis.
- Extended studies to cultured dopaminergic neurons.
- **Discovered that both MPP⁺ and 6-OHDA induce markers of ER stress.**

Reportable Outcomes

- An abstract describing these studies will be presented at the Society for Neuroscience Annual Meeting.
- Data obtained from these experiments will be submitted and shared via the guidelines provided by the National Center for Biotechnology Information Expression Omnibus.
- These studies are being prepared for publication.

Conclusions

The central hypothesis of these studies is that changes in gene expression underlie much of the damage that ultimately leads to the death of dopaminergic neurons after treatment with 6-OHDA or MPP⁺. Using DNA micro array technology we determined that both of these neurotoxins induce ER stress although not to the same degree. Identification of key genetic components of this response may suggest new points of intervention. Taken together, these experiments will help clarify the molecular mechanisms associated with 6-OHDA and MPP⁺ toxicity and might aid in developing novel therapeutic avenues to pursue relevant to PD.

References

Lotharius J., Dugan L.L., O'Malley K.L. (1999) Distinct mechanisms underlie neurotoxin-induced cell death in cultured dopaminergic neurons. *J. Neurosci.* 19: 1284-1293.

Oh Y.J., Wong S.C., Moffat M., O'Malley K.L. (1995) Overexpression of Bcl-2 attenuates MPP+, but not 6-OHDA-induced cell death in a dopaminergic neuronal cell line. *Neurobiol. Dis.* 2: 157-167.

Choi WS, Yoon SY, Oh TH, Choi EJ, O'Malley KL, Oh YJ. Two distinct mechanisms are involved in 6-hydroxydopamine- and MPP+-induced dopaminergic neuronal cell death: role of caspases, ROS, and JNK. *J Neurosci Res.* 1999 Jul 1;57(1):86-94.

Kaufman RJ. (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev.* 13:1211-33.

Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K, Suzuki T. (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet.* 25:302-5.

Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell.* 105:891-902.

Figure 1

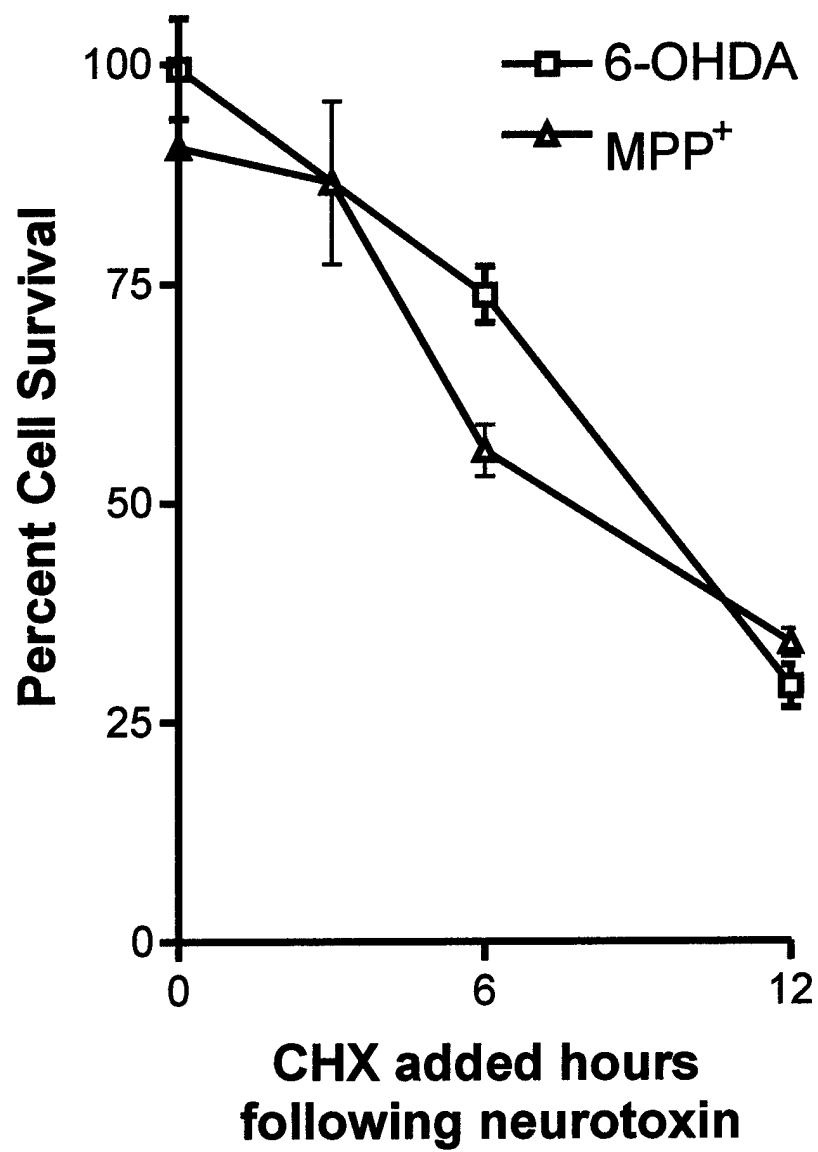


Figure 2

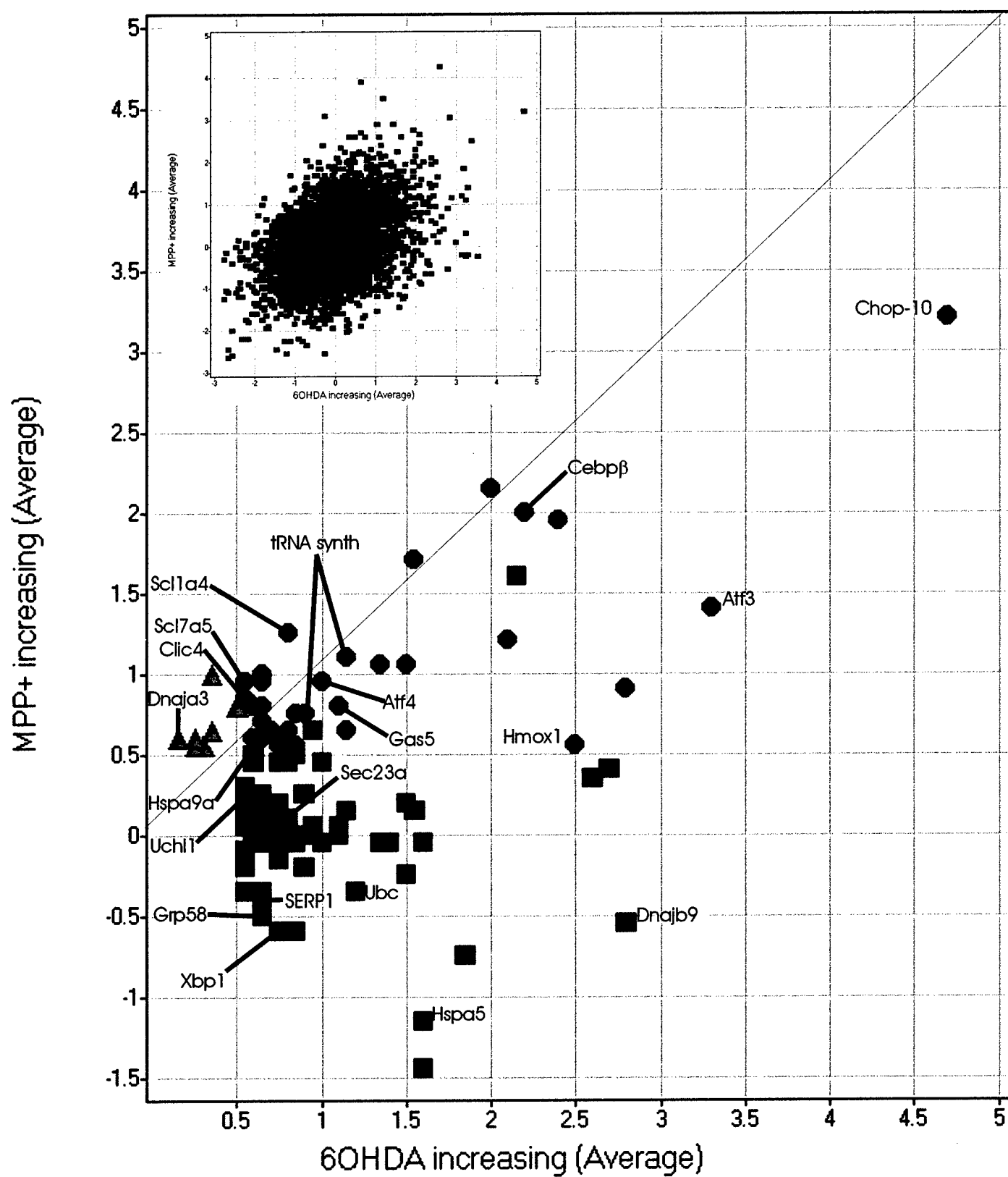


Figure 3A

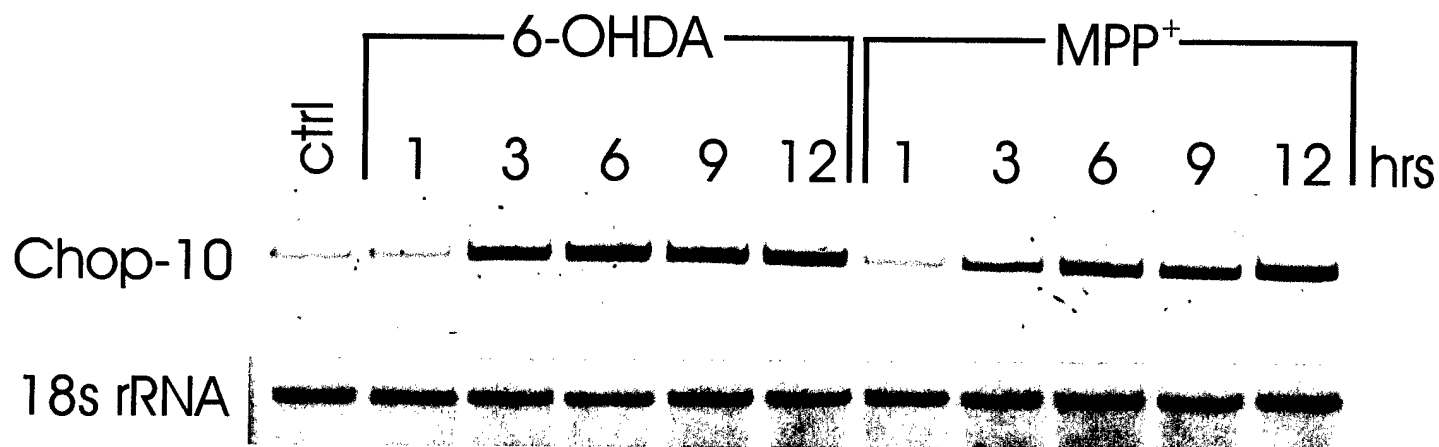


Figure 3B



Figure 4A

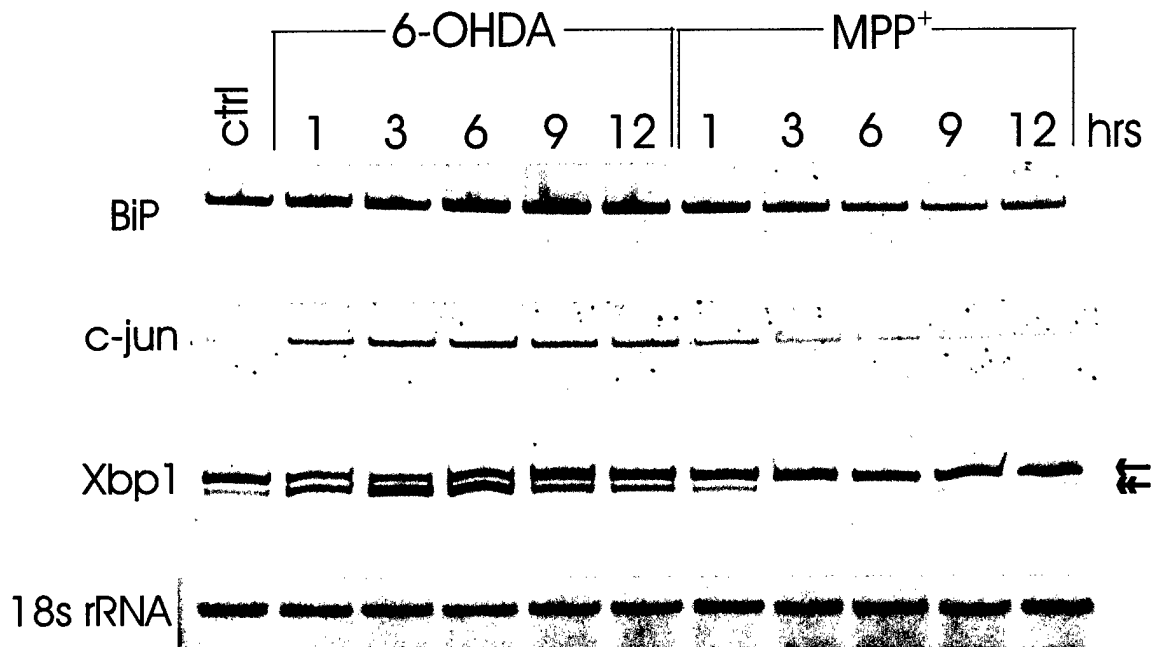


Figure 4B

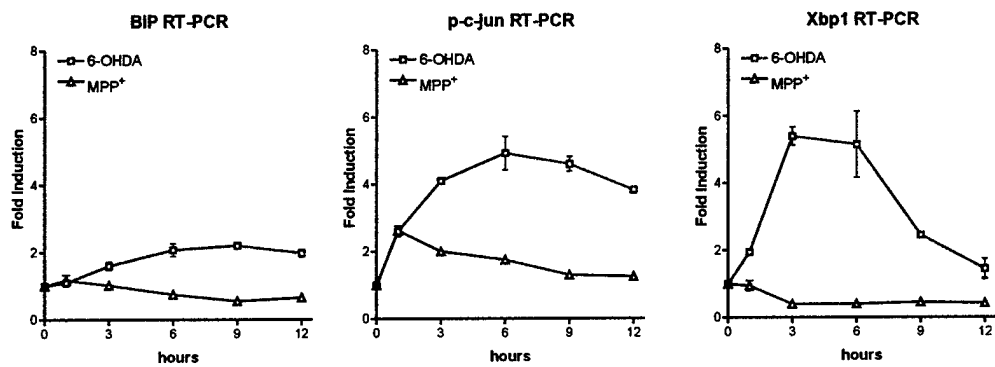


Figure 5A

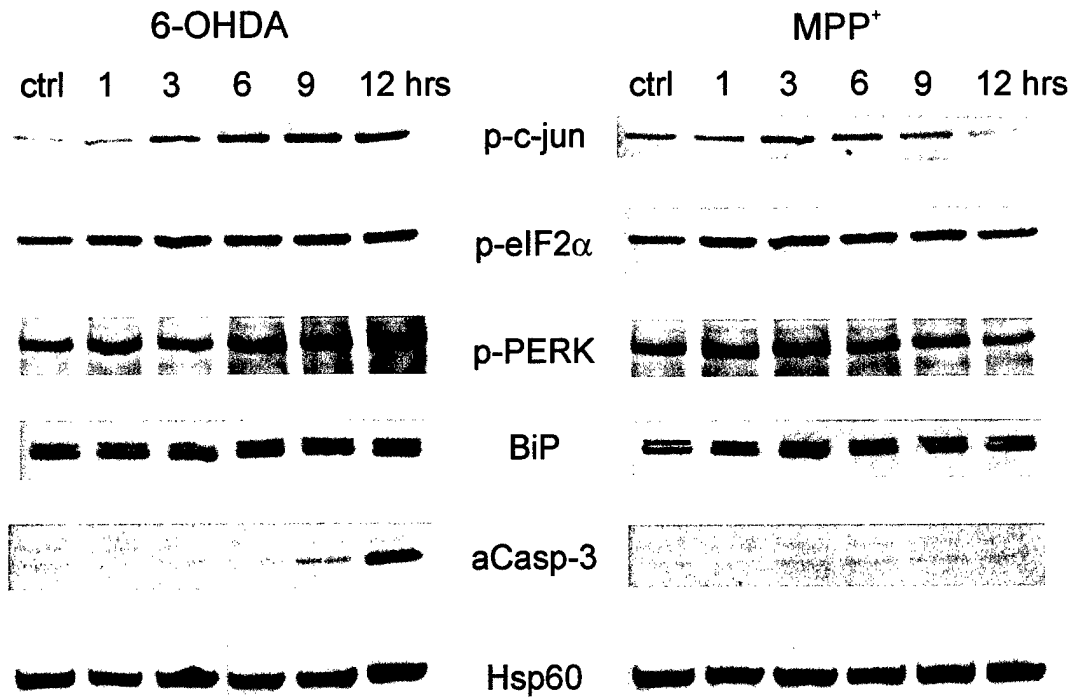


Figure 5B

